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Ibrutinib-A double-edge sword in cancer and autoimmune disorders

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Abstract

Targeted therapies have appeared as new treatment options for several disease types, including cancer and autoimmune disorders. Of several targets, tyrosine kinases (TKs) are among the most promising. Overexpression of TKs provides a target for novel therapeutic agents, including small molecule inhibitors of tyrosine kinases (TKI). Ibrutinib (PCI-32765) is a TKI of Bruton's tyrosine kinase (Btk), a key kinase of the B-cell receptor signaling pathway that plays a significant role in the proliferation, differentiation and survival of B cells. In addition to inhibitory effects, recent studies have shown that ibrutinib has multiple immunomodulatory effects. It binds covalently to IL-2 inducible tyrosine kinase (Itk) in T lymphocytes and suppresses the survival of T-helper (Th) 2 cells. This changes the balance of Th1/Th2 cells toward Th1 subset, which are the main immune cells targeting tumor cells. The dual activity of ibrutinib has paid a great attention and several studies are evaluating the anti-tumor and immunomodulatory effects in cancer, autoimmune disorders and infectious diseases. In this article we review the inhibitory and immunomodulatory effects of ibrutinib in B-cell malignancies, autoimmune diseases and infections, as well as the communication between the Ror1 receptor tyrosine kinase and BCR and effects of ibrutinib on this crosstalk.

Introduction

During the recent years, several targeted-therapy agents have been developed. These agents are more specific for tumor cells than normal cells [1]. Small molecule inhibitors (SMIs), including kinase inhibitors are potent agents for the treatment of cancer and autoimmune diseases [2-9]. These agents induce apoptosis of tumor cells through blocking molecules involved in cell survival [9].

Among several SMIs, ibrutinib (PCI-32765) has emerged as the first-in-class SMI targeting Bruton's tyrosine kinase (Btk) and received FDA approval for the treatment of mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL) [10] and recently was approved for the treatment of Waldenström's Macroglobulinemia on January 29, 2015.

Btk is involved in the B-cell receptor (BCR) signaling and is vital for many aspects of the B-cell development. Several studies have shown that ibrutinib binds to Btk with high affinity and leads to inhibition of BCR signaling, reducing the activation of malignant B cells and B cells involved in autoimmunity and infectious disease pathogenesis [11-14].

BCR upregulation and its signaling pathway is a hallmark of the pathophysiology underlying MCL [15-16], CLL [17-21], diffuse large B-cell lymphoma (DLBCL) [22-23], and other B-cell malignancies by continuous stimulation of B cells through binding to a variety of antigens [24-25]. Mutations in the *Btk* gene result in an immunodeficiency disorder named Bruton's agammaglobulinemia or X-linked agammaglobulinemia (XLA), where patients have decreased, or lack of immunoglobulins and increased risk of infections [26].

Ibrutinib treatment of leukemic CLL cells inhibits BCR signaling downstream of Btk with a decline in pro-survival cytokines and apoptosis of leukemic cells [27]. The safety and efficacy of ibrutinib in CLL and MCL might also suggest the use in other B-cell malignancies, autoimmune diseases and infections [10, 28-29]. Several trials are assessing ibrutinib in malignant B-cell disorders, including CLL, DLBCL, alone or in combination with other drugs (Table 1) [22].

Several studies have demonstrated that ibrutinib targets other members of Tec family proteins. Ibrutinib has been shown to block IL-2 inducible tyrosine kinase (Itk) in T cells. T helper (Th) 1 cells, also express another kinase called resting lymphocyte kinase (Rlk or Txk). Ibrutinib inhibits Itk in T cells but only Th1 cells will survive due to the activation of the Rlk survival pathway

[14]. Therefore, the balance between Th1/Th2 cells will skew toward Th1 type, which is important for tumor cell deletion, cells infected with intracellular pathogens and for the prevention of production of autoreactive antibodies production. The immunomodulatory activity and inhibition of Btk suggest that ibrutinib may be an interesting agent for targeted therapy.

This article gives an overview of four major topics and recent findings, including 1) structure and function of Btk and Itk in BCR and T-cell receptor (TCR) signaling, 2) preclinical and clinical studies on the effects of ibrutinib in the treatment of malignant and autoimmune disorders, 3) communication between the Ror1 receptor tyrosine kinase and BCR and effects of ibrutinib on the crosstalk, and 4) novel roles of ibrutinib as a modulator of the immune system and the therapeutic applications in cancer, autoimmunity and infections.

Btk structure and function

Btk belongs to the Tec family of tyrosine kinases, which includes Btk, Itk, Bmx, Rlk, and Tec (Fig. 1). Tec family is the second largest family of non-receptor kinases after Src family. Btk structure consists of pleckstrin homology (PH), Tec homology, Src homology (SH)3, SH2 and SH1 domains [30]. The SH1 domain is the catalytic part of Btk (Figs. 1, 2).

Btk is expressed in myeloid- and B-lymphoid cells but not in T cells and plasma cells [31-34]. Btk is important for several steps in the B-cell lineage development, including B-cell ontogeny, differentiation and survival [30, 35-36]. Btk binds to several cytosolic proteins to transduce signals to transcription factors (Fig. 2).

B-cell development is strictly regulated by a balance between self-governing mechanisms (B-cell antigen receptor or pre-BCR/BCR), the bone marrow microenvironment and key cytokines, including interleukin (IL)-7. IL-7 is released by bone marrow stromal cells, leading to a properly assemble of pre-BCR. Pre-BCR is of importance for the further development of B cells and finally, the mature BCR is responsible for the mature B cell survival.

The BCR is composed of the surface immunoglobulin, as the antigen binding site coupled with the signal transduction molecules, Igα/Igβ dimers (CD79A, CD79B) (Fig. 3). Stimulation of the BCR by antigen induces aggregation of BCR components, BCR oligomerization and BCR cluster

formation [37] that leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the cytoplasmic tails of Igα and Igβ. The latter is then activating Lyn and other Src family kinases (Fyn and Blk), which in turn activate Syk, Btk and phosphatidylinositol-3 kinase (PI3K) isoforms.

Following antigen binding to BCR, phosphatidylinositol-3, 4, 5- triphosphate (PIP3) levels are increased, leading to the recruitment of Btk to the cell membrane. Several proteins of the Src family, including Lyn and Syk, phosphorylate Btk at the tyrosine residue (Tyr) 551 at the activation loop of the kinase domain. Phosphorylation of Tyr 551 leads to autophosphorylation of Tyr 223 in the SH3 domain of Btk followed by activation of Btk kinase. The adapter proteins, including phospholipase Cγ2 (PLCγ2) and BLNK then translocate to the cell membrane and both will be phosphorylated by Btk [38]. Calcium will be mobilized and increased in the cytoplasm and protein kinase C (PKC) as well as nuclear factor-κB (NF-κB) activation occurs. Finally, NF-κB activates several genes such as various cytokines, growth factors and receptor tyrosine kinases (RTKs) that control activation, proliferation, differentiation, and survival of B cells [30, 39]. Btk may play a crucial role in B-cell signaling and prevention of apoptosis of malignant B cells [40].

Btk has critical roles in several hematopoietic cells signaling pathways. Due to the role of Btk in the BCR and cytokine receptors signaling, it is an attractive protein in targeted therapies of B-cell disorders, including malignant and autoimmune diseases.

Overview of ibrutinib pharmacokinetics (PK) and pharmacodynamics (PD)

SMIs of tyrosine kinases (TKIs) have been classified into five classes. Each type has different effects on the PK and PD of the drugs. Type 1 TKIs are the most frequent type and are adenosine 3-phosphate (ATP)-competitors. These inhibitors detect the active structure of the substrate and mimic ATP [41].

Type II targets the inactive conformation of kinases and occupies an extra hydrophobic pocket created by a conserved Asp–Phe–Gly (DFG) amino acid sequence. These inhibitors are more effective than type I and have better selectivity and slower off-rate [42-43]. Type III inhibitors bind to the allosteric site of the substrate near to the ATP binding pocket and are highly selective and specific for the target. Type IV TKIs forms an irreversible bond to the active site of the target

via binding to a cysteine (Cys) residue. Epidermal growth factor receptor (EGFR) inhibitor HKI-272 and ibrutinib belong to this group [43]. Type V inhibitors are classified as a new type, and a few TKIs has been classified in this group. These inhibitors are bivalent compounds that target two distinct regions of the kinase. This bivalent binding will significantly increase affinities and enhanced selectivity of TKI for the targeted kinase. These new type of inhibitors might be the most useful drugs for personalized medicine and investigating signal transduction.

Targeting BCR signaling has several therapeutic advantages. BCR signaling could be inhibited at different levels, for instance by antigen blocking, interrupting antigen binding or inhibition of BCR downstream components (Fig. 3).

Currently, the lead compounds for targeting BCR signaling are kinase inhibitors that target the BCR or associated kinases as PI3K and spleen tyrosine kinase (Syk) (Fig. 3). Most investigations have focused on the development of ATP-competitive drugs that target the ATP-binding pocket of protein kinases. However, there are several challenges in the development of such inhibitors. Poor selectivity and competition with the ATP-binding pocket, due to high concentrations of endogenous ATP, have been observed and are considered as the major obstacles [3].

Irreversible kinase inhibitors (type IV) such as ibrutinib are attractive alternatives for ATP-competitive drugs. These inhibitors show high selectivity, specificity and prolonged PD.

Ibrutinib is the first covalent inhibitor of Btk reported by Pan et al. in 2007 and was developed to treat rheumatoid arthritis (RA) [44]. It has been developed from the reversible Btk inhibitor PCI-29732. Ibrutinib is 16-fold more potent than the primary SMI PCI-29732 for inhibition of Btk. It binds covalently to Cys 481 in the ATP-binding pocket of Btk. The molecular formula is $C_{25}H_{24}N_6O_2$, with a molecular weight of 440.5 g/mol. Ibrutinib has an IC_{50} of 0.5 nM for Btk [44].

PK studies have shown that ibrutinib is rapidly absorbed and eliminated after oral administration within 1–2 hours (h) and binds to Btk for more than 24 h [10]. The drug is metabolized primarily by the liver. Several clinical trials have shown that the maximum tolerated dose for ibrutinib was not reached in both doses of 560 and 420 mg/day that are recommended for the treatment of MCL and CLL, respectively [13].

Ibrutinib binds to the non-phosphorylated Btk and stabilizes the inactive conformation by internalizing Tyr 551 and prevents the phosphorylation by other kinases such as Src. Ibrutinib inhibits other kinases, including Blk, Bmx, EGFR, Itk, and Janus kinase (JAK) 3 (IC_{50}=0.5, 0.8, 5.6, 10.7, and 16.1 nM, respectively) [45]. These kinases have a cysteine residue in the homologous location to Btk [30, 45]. Ibrutinib has shown to be 1000-fold more selective for inhibition of BCR signaling in B cells over TCR signaling in T cells.

In spite of the several disadvantages of multi-targeted SMIs [3], the perspective of ibrutinib is mainly that of a special agent with dual effects. These positive effects will be discussed later. Moreover, Btk is expressed by other cells at lower levels than B cells [46-50] and therefore will not be highly affected by ibrutinib.

Preclinical studies of ibrutinib in cancer

Several preclinical studies have shown inhibition of BCR by ibrutinib [45], and direct cytotoxicity in DLBCL, MCL, hairy cell leukemia (HCL), and primary CLL cells [51-55]. Inhibition of cytokines and integrin associated signaling and leukemic cell migration have also been demonstrated by *in vitro* and *in vivo* models [56-57].

A rapid downregulation of BCR and NF-κB signaling pathways *in vivo* in CLL cells has also been noted during ibrutinib treatment [58]. Ibrutinib decreased the phosphorylation of PLCγ2 and Erk as well as reduced nuclear expression of NF-κB p50. Ibrutinib significantly inhibited the expression of CD69 and CD86 in tumor cells, independent of IgVH mutational status [59-60] or chromosome 17p deletion [61]. A stronger inhibition of BCR signaling in lymph node CLL cells was shown to be correlated with a higher rate of nodal response. Moreover, on-target effects of Btk inhibition by ibrutinib efficiently prevented pathways that stimulate tumor cell activation and proliferation *in vivo* [58].

Ibrutinib has inhibitory effects on malignant plasma cells. Combination treatment in multiple myeloma (MM) with ibrutinib, bortezomib and lenalidomide has shown significant increase in the observed cytotoxic effects. Ibrutinib prevented phosphorylation of the serine residues at

position 536 of the p65 subunit of NF-κB and blocked nuclear translocation and downregulated the anti-apoptotic proteins Bcl-xL, FLIP and survivin in myeloma cells [62].

Effects of ibrutinib alone or in combination with bortezomib have been studied on Raji and Ramos Burkitt's lymphoma cell lines [63]. Treatment with ibrutinib (0.5-6.0 μmol/L) and bortezomib (10-80 nmol/L) inhibited cell proliferation and survival in a dose- dependent and time-dependent manner with synergistic effects. Ibrutinib and bortezomib also inhibited the expression of intracellular NF-κB, Bcl-xL and c-IAP1 proteins and upregulated the expression of caspase-3.

Ibrutinib efficacy has also been examined using breast cancer cell lines [64] and was shown to significantly reduce phosphorylation of the receptor tyrosine kinases EGFR, human epidermal growth factor receptor (HER) 2 and HER3 and suppressed the Akt and mitogen-activated protein kinases (MAPK) signaling pathway in $HER2^+$ cells. Moreover, treatment of $HER2^+$ cell lines with ibrutinib significantly decreased cell survival with IC_{50} values at nanomolar concentrations. Combination of the PI3K/mammalian target of rapamycin (mTOR) inhibitor BEZ235 with ibrutinib synergistically decreased cell viability of $HER2^+$ breast cancer cells. The data suggested a therapeutic potential of ibrutinib in breast cancer and that the combination of PI3K inhibitors with ibrutinib might be considered as an effective approach in the treatment of breast cancer with activated ErbB receptors [64].

Synergistic effects of ibrutinib and dual mTORC1/2 inhibitors have been examined *in vitro* using DLBCL cells [65]. The combination of ibrutinib and AZD2014, an mTOR inhibitor has strong synergistic effects in killing activated B-cell-like (ABC) subtype of DLBCL (ABC-DLBCL) cell lines. Simultaneous inhibition of Btk and mTOR induced apoptosis of DLBCL cell lines both *in vitro* and *in vivo* as well as tumor regression in a xenograft model. Inhibition of cap-dependent translation and the inhibition of NF-κB/IL-10/signal transducer and activator of transcription (STAT) 3 autocrine loop was shown to be the main mechanism of apoptosis and combined with disruption of the Btk and mTOR signaling pathways [65].

The synergistic, additive and antagonistic drug combinations have been studied in ABC-DLBCL using a high-throughput screening method [66]. Combination of ibrutinib with a wide range of

compounds, including inhibitors of PI3K/Akt, mTOR, other BCR pathway, Bcl-2 family SMIs, and chemotherapies that are the standard treatments for DLBCL has shown favorable results [66].

Combination of ibrutinib and idelalisib (PI3Kδ inhibitor) has shown synergistic effects on inhibiting BCR signaling and targeting integrin-associated adhesion and migration of the tumor cells to the lymphoid organs. The combination results in malignant cells mobilization from the supporting niches into the blood and finally deprives the tumor cells of vital growth and survival signals [67].

Clinical studies of ibrutinib in cancer

Ibrutinib has been studied in several clinical settings for the treatment of lymphomas and leukemias (Tables 1, 2).

Preliminary clinical trials in malignancies (refractory CLL, DLBCL, MCL, follicular lymphoma (FL), and marginal zone lymphoma (MZL)) indicated a proper safety profile at doses that achieved a higher than 95% enzyme occupancy and a high overall response rate (ORR) [55, 68-70].

In a phase I trial, 56 patients with relapsed/refractory B-cell malignancies were treated with ibrutinib (Clinical trials.gov identifier: NCT00849654, Table 2). It was well-tolerated in doses up to 12.5 mg/ kg once/day [71]. The major side effects were rash, fatigue, diarrhea, nausea, and dyspepsia. The occupancy of Btk by ibrutinib was determined *ex vivo* by using the fluorescent probe PCI-33380. Complete Btk occupancy in the blood was observed at 2.5 mg/kg four times daily (q.i.d). PK data indicated that ibrutinib was rapidly absorbed with a half-life of 6.3–10.8 h. Btk occupancy was continued for 24 h. Of 50 evaluated patients, ORR and progression free survival (PFS) were 60% and 13.6 months, respectively [71].

In an open-label, phase Ib/II trial (NCT01105247 and NCT01217749, Table 2), ibrutinib was evaluated as initial therapy for elderly patients with CLL (n=29) or small lymphocytic leukemia (SLL) (n=2) [72]. Previously untreated patients older than 65 years with symptomatic CLL or SLL requiring therapy were enrolled. Patients received a 28 days cycle of 420 or 840 mg ibrutinib. However, in future studies only 420 mg was used as this dosage had the same effect as

840 mg. The primary endpoint was the safety of the dose-fixed regimen. Toxicity was mostly of grade 1-2, including diarrhea, nausea, fatigue and grade 3 infections, neutropenia, and thrombocytopenia. After a median follow-up of 22.1 months, 22 of 31 patients achieved an objective response (95%, CI=52.0-85.8), four patients showed a complete response (CR), one patient had a nodular partial response (PR), and 17 patients presented a PR [72].

Ibrutinib was evaluated in a phase II study of refractory MCL patients (n=111) (NCT01236391, Table 2) [29]. Eighty six percent of patients who had intermediate/high-risk disease were treated with ibrutinib at 560 mg q.i.d. The ORR, CR and PR rates were 65.8%, 17% and 49%, respectively. The most important side effects were mild atrial fibrillation, skin infections, nausea, diarrhea, pneumonia, abdominal pain, and fatigue [29].

During the median follow-up (15.3 months, range 1.9 to 22.3 months), 46 patients continued treatment, and 65 discontinued treatment. Discontinuation was due to disease progression, patient or investigator decision, and adverse effects (pneumonia, subdural hematomas, sepsis, cardiac arrest, elevated bilirubin level, respiratory failure, and metastatic adenocarcinoma). The results of this study led to the approval of ibrutinib by the FDA for the treatment of MCL with at least one prior therapy as a single agent [30]. Three months later, (February 12, 2014) ibrutinib was approved for the treatment of CLL, who have received at least one prior therapy [10].

Several trials are testing ibrutinib in combination with monoclonal antibodies (mAbs) and chemotherapy agents in B-cell malignances (Table 1, 2).

In a phase Ib study (NCT01292135, Table 2), the safety and efficacy of ibrutinib were investigated in combination with chemo-immunotherapy in relapsed/refractory CLL patients [73]. Patients received bendamustine and/or rituximab (anti-CD20 antibody) (BR), cyclophosphamide, fludarabine, and rituximab (FCR) with ibrutinib (420 mg) until disease progression or toxicity were observed. Due to the lack of fludarabine-untreated patients, the recruitment of patients for FCR-ibrutinib arm was stopped. No toxicity in cycle one was observed in patients treated with BR-ibrutinib and FCR- ibrutinib (primary end point). The ORR was 93.3% in BR-ibrutinib treated patients, and CR was 16.7% at the early stages of treatment that improved with the extension period (40%). 86.3% and 70.3% of patients showed no sign of disease progression after 12 and 36 months, respectively [73].

In a phase Ib study (NCT01569750, Table 2) combination of ibrutinib with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has been evaluated for treatment-naive patients (n=33) with CD20-positive B-cell non-Hodgkin lymphoma (B-NHL) [74]. The primary endpoint was to identify a suitable dose of ibrutinib when combined with a standard R-CHOP regimen. The secondary endpoint was ORR and PK. Patients received 280, 420, or 560 mg ibrutinib per day in combination with R-CHOP regimen every 21 days. The maximum tolerated dose was not achieved and 560 mg per day was the recommended dose for subsequent studies. The most important side effects (grade 3) were neutropenia (73%), thrombocytopenia (21%), febrile neutropenia, and anemia (18% each). The most often described adverse effects included febrile neutropenia (18%) and hypotension (6%). Thirty of 32 patients treated with one or more doses of combination regimen achieved an OR. All 18 patients with DLBCL who received the 560 mg dose showed an OR. Five of seven patients (71%) with the germinal center B-cell-like subtype and two patients (100%) with the non-germinal center B-cell-like subtype had a CR. R-CHOP did not change PK of ibrutinib, and ibrutinib did not affect the PK of vincristine [74].

In a recent ongoing open-label, phase III trial (NCT01578707, Table 1), 391 patients with relapsed or refractory CLL or SLL were treated with daily ibrutinib or ofatumumab (anti-CD20 antibody) [28]. PFS was the primary end point, and secondary end point was OS. Ibrutinib treatment showed significant improvement of PFS rate (88% at 6 months) compared to ofatumumab group with a median of 8.1 months, but the median duration was not reached in the ibrutinib treated patients. Ibrutinib also improved OS compared to ofatumumab group (90% vs. 81% after 12 months). The ORR was 42.6% in the ibrutinib group compared to 4.1% in ofatumumab group ($P<0.001$) [28].

Safety and activity of ibrutinib plus rituximab have been evaluated in 40 patients with high-risk CLL [75]. In this phase II study patients with cytogenetic abnormalities (deletion 17p, TP53 mutation or 11q deletion) or a short PFS (<36 months) after previous first-line chemoimmunotherapy were enrolled. Patients with symptomatic disease requiring therapy received 28-days cycles of once-daily ibrutinib (420 mg) combined with rituximab (375 mg/m(2)

, every week during cycle one and then once per cycle until cycle six) together with daily single-agent ibrutinib (420 mg) until disease progression or unexpected toxicities. The primary endpoint was PFS in an intention-to-treat population [75].

Grade 1-2 diarrhea (25%), bleeding events (33%), nausea or vomiting (38%), and fatigue (18%) were noted in patients. Five patients (13%) showed grade 3 infections (lung infections, upper respiratory tract infection, sepsis, and mucositis), no grade 4 or 5 infections were observed and one patient had a grade 4 neutropenia [75]. In this study with a follow-up of 16.8 months (median), 31 patients continued in the trial (14 of the 20 with del [17p] or TP53 mutation) and nine patients (22.5%) discontinued. Of discontinued patients, two died during the trial and six died after discontinuation of the study. One of the patients who died during the trial had an infectious complication that was unrelated to the study during the second cycle of study treatment, and one patient died during the remission due to an unknown reason. The other six patients died from infectious problems or after disease progression and two patients discontinued treatment due to the drug toxicity after the cycles five and two, respectively. Two patients discontinued the treatment due to resistant pneumonia and obstructive pulmonary complications [75].

The combination of ibrutinib with bendamustine and rituximab is testing in a phase III study in relapsed CLL/SLL patients (the HELIOS trial) [76]. This trial is investigating if ibrutinib in combination with bendamustine and rituximab (BR) has significant benefits over BR alone. All patients receiving BR (six cycles) were randomized 1:1 to placebo or ibrutinib 420 mg/day. Treatment with ibrutinib and placebo was started in parallel with BR and continued until disease progression or unacceptable side effects were observed. The primary end point of this trial was PFS and the secondary end points were safety, ORR, OS, rate of minimal residual disease-negative remissions, and patient-reported outcomes [76].

Itk structure and signaling in T cells

Itk (Emt, Tsk) belongs to the Tec family proteins and expresses in T lymphocytes, natural killer and mast cells [32, 77-83]. Itk has similar structural characteristics as Btk, including the common SH1, SH2, SH3, PH domains, Zinc binding motif (Btk homology, BH), and a proline-rich region

(PRR) [84]. The PH domain is important to recruit the Itk kinase to the cell membrane and mediates the interaction of Itk with membrane bound phospholipids [85]. The SH3 domain is involved in the regulation of Itk and the interaction with PRR regions of other signaling proteins. The SH2 region is important for Itk localization and activation. It is critical for transphosphorylation and interaction of Itk with TCR-induced signaling proteins associate with the cell membrane. The SH2 domain also regulates the enzymatic activity of Itk. Disruption of this domain induces lack of kinase activity of Itk in contrast to the role of SH3 domain [86]. Itk is involved in TCR signaling as well as in Th1/Th2 balance and specifically regulates the function of Th2 lymphocytes. Lack of Itk has profound effects on Th2 cells [87].

Stimulation of TCR promotes localization of Itk to the TCR/CD3/CD28 complex activating Src and Lck kinases followed by phosphorylation of CD3 [85]. Simultaneously, Zeta-chain-associated protein kinase 70 (ZAP-70) binds to the activated CD3 and then Lck kinase phosphorylates ZAP-70 and activates the adaptor proteins, including the linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76 kDa (SLP-76) [88]. Subsequently, Itk is recruited to the cell membrane following activation of PI3K and accumulation of PIP3. In the cell membrane, Itk interacts with the pSLP-76/LAT complex via the Itk SH3 and SH2 domains following phosphorylation of Itk at Tyr 511 by Lck. Phosphorylated Itk activates the downstream signaling molecule PLCγ1 [84], which hydrolyses phosphatidylinositol-4,5-diphosphate (PIP2) to produce Inositol-3-phosphate (IP3) and diacylglycerol (DAG) [89], increasing calcium flux, Erk activation, actin reorganization (TCR-induced actin polymerization), and cytokine release [90].

Several Itk-SMIs are in preclinical development, but none has entered clinical trials. Considerable efforts have been made to develop Itk-SMIs to treat autoimmunity and allergy. The majority of Itk inhibitors lack selectivity and specificity to effectively suppress T-cell functions. However, a series of indazoles that show sub-nM inhibitory potency against Itk with strong cellular activity and kinase selectivity have recently been produced [91].

Immunomodulatory role of ibrutinib

The broad activity of ibrutinib may have therapeutic indications through binding to the Itk kinase, which is of importance for Th2 cells. Ibrutinib has been shown to have immunomodulatory activity by inhibiting Itk in T cells derived from patients with CLL. Ibrutinib bond covalently to Itk at Cys 442 and occupy the active site followed by T cells inactivation [14]. However, in contrast to Th1 cells, Th2 cells were sensitive to ibrutinib and demonstrated reduced production of IL-4 in Th2 naive cells but no effects on IFN-γ production [14].

Ibrutinib also inhibited Itk autophosphorylation at Tyr 180 in a dose-dependent manner and inactivated the downstream molecules IkBα, Junβ, STAT6, and nuclear factor of activated T-cells (NFAT) in primary $CD4^+$ cells as well as in Jurkat cells. Junβ and STAT6 are critical in the IL-4 signaling of Th2 cells. However, phosphorylation of upstream molecules such as Lck, ZAP70 and LAT did not change. Moreover, a significant decrease in TCR-induced phospho-PLCγ1-Tyr 783 dephosphorylation and inactivation in CD3/CD28-stimulated $CD4^+$ T cells from CLL patients treated with ibrutinib was observed [11].

Resting lymphocyte kinase (Rlk) plays a redundant role for Itk in Th1 cells, but neither Th2 cells nor Jurkat cells expresses Rlk kinase. Jurkat cells transduced to express Rlk showed that the NFAT transcription factor, a downstream molecule of the TCR signaling pathway, was protected in the transfected cell line by the inhibitory effects of ibrutinib. Moreover, the intracellular calcium was significantly increased in the parent or Rlk untransfected Jurkat cells indicating that Rlk compensates for ibrutinib-inhibited Itk and provides an alternate activation platform for Itk/Rlk-expressing cells. $CD4^+$ T cells from healthy controls and CLL patients showed a significant outgrowth of Th1 cells after ibrutinib treatment [14].

The immunomodulatory effects of ibrutinib are interesting, and further investigations are warranted.

Preclinical studies of ibrutinib in autoimmune diseases and infections

Any compound that inhibits BCR signaling may inactivate B cells in any diseases. The main goal of ibrutinib development was to block Btk and B-cell proliferation in RA and systemic lupus erythematous (lupus) (SLE) [45, 92]. Ibrutinib-related compounds inhibited the disease development in a murine RA model [44, 93]. Ibrutinib prevented collagen-induced arthritis

(CIA), as well as the production of autoantibodies and prevented the development of kidney disease in MRL-Fas (lpr) mice [45]. A Btk inhibitor (CGI1746) has been shown to prevent B cell proliferation and decrease the production of collagen-induced autoantibodies [94]. Btk inhibition diminished production of pro-inflammatory cytokines as tumor necrosis factor (TNF) α, IL-1 and IL-6, suggesting multiple targets of Btk inhibition in autoimmune diseases [94].

The inhibitory effects of ibrutinib have been studied in several animal models, including reversed passive anaphylactic reaction, passive cutaneous anaphylaxis and collagen antibody-induced arthritis. High efficacy of ibrutinib in CIA models and animal models with immune-complex disorders was observed. Data suggest that ibrutinib may target other pro-inflammatory cells such as macrophages, mast cells and monocytes [92-93]. A link between Toll-like receptor (TLR) 9-induced Btk activation has been described in PIR-B-deficient B-1 cells (paired immunoglobulin-like receptor-B), inducing high titers of autoantibodies and autoimmunity [95]. In a mouse model with Btk overexpression in B cells, spontaneous formation of germinal centers, a significant increase of plasma cells, production of anti-nuclear autoantibodies (ANA), and induction of SLE-like autoimmune disease was noted. These autoimmunity signs were significantly decreased in transgenic mice over-expressing a kinase-inactive Btk mutant. Treatment with ibrutinib reduced germinal center formation, decreased the number of plasma cells and inhibited the normal function of B cells [13]. Ibrutinib treatment also decreased humoral and cellular immunity and lupus nephritis in lupus-prone B6.Sle1 and B6.Sle1.Sle3 mice [12, 93].

In a preclinical study, a dose-dependent occupancy of Btk (85–90%) was observed in the spleen of the lpr lupus mice 3 h after the final dose. The level of Btk inhibition was associated with the efficacy. Ibrutinib prevented the development of kidney disease measured as inhibition of production of autoantibodies such as anti-dsDNA antibodies, proteinuria and increase in blood urea. A significant decrease was noted in interstitial nephritis, perivascular inflammation and glomerulonephritis. Moreover, a complete healing of the disease was also observed [30, 96].

Ibrutinib has also been shown to prevent osteoclast-mediated bone loss [97]. Bone-resorbing osteoclasts play a crucial role in normal bone homeostasis and disorders as osteoporosis and RA. Tec family is essential for the differentiation of osteoclasts and inhibition of Btk might prevent the bone loss. Ibrutinib may inhibit osteoclastic bone resorption by suppressing osteoclast differentiation and function, downregulating the expression of NFATc1, the main transcription

factor for osteoclastogenesis and disrupts the formation of the actin ring in mature osteoclasts. Ibrutinib could also inhibit bone loss in a receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoporosis mouse model [97].

A positive clinical effect of ibrutinib is not only mediated through inhibiting B cells, but also it is by changing the Th1/Th2 balance toward Th1 cells. Ibrutinib may be effective in inhibiting the growth of intracellular pathogens. Ibrutinib treatment has shown a significant increase of Th1 cells as well as memory T cells in mice infected with intracellular pathogens [14].

In an interesting study, CLL cells were engrafted into mice and treated with ibrutinib or vehicle. Mice were then infected with Listeria monocytogenes expressing the immunodominant chicken ovalbumin (OVA) protein. Analysis of T cells showed a significant lower frequency of OVA-specific $CD8^+$ T-cell response in the leukemic vehicle treated control group as compared to ibrutinib treated mice. Ibrutinib not only restored the magnitude of the immune response, but also boosted the immune system against the infection. Non-leukemic mice and ibrutinib-treated leukemic mice cleared the infection by day 8, but in leukemic mice treated with vehicle Listeria contamination were noted within the liver. Rechallenge of ibrutinib treated mice with Listeria indicated no loss of a functional immunologic memory as determined by the measurement of $CD62L^+/CD4^+$ and $CD62L^+/CD8^+$ cells [14].

The human immunodeficiency virus-1 (HIV-1) Nef virulence protein interacts with host cell-signaling molecules. Nef binds to the SH3 domain of Src family kinases (Hck, Lyn, and c-Src), which are activating kinases of importance for viral activity, including infectivity, replication and HLA down-regulation [98]. Itk, Bmx and Btk but not Tec or Txk are present in HIV target cells, and they are linked to HIV-1 infectivity [99]. Nef interacts with intracytoplasmic kinases of the Tec enzyme family and maybe a link between HIV-1 and Itk during the viral life cycle. A specific/selective SMI of Itk (BMS-509744) suppressed the wild-type HIV-1 activity, but not the Nef-defective mutant. Nef induced activation of Itk in transfected cells that were sensitive to SMI treatment. Ibrutinib might have the same effects as BMS-509744 and not only block the HIV interaction with cells, but also inhibit the viral infectivity and spread through activation of Th1 cells [99].

Itk also regulates T cell signaling by organizing actin polymerization, polarization, recruitment of kinases and adapter proteins as well as controlling different steps of HIV-1 infectivity, including virion assembly and release [100]. Itk and group-specific antigen (Gag) protein colocalized at the membrane and are concentrated at sites of F-actin accumulation and lipid rafts in infected T cells. Itk specific SMIs inhibit F-actin capping, disturb Gag-Itk colocalization, block virus particle release, and reduce HIV duplication in human $CD4^+$ T cells. Targeting Itk by SMIs such as ibrutinib and other selective Itk inhibitors might prevent HIV-1 egress and control HIV infection [100].

Itk kinase activation is also shown to play a central role in the replication of influenza virus in T cells [101]. Treatment of influenza-infected patients with ibrutinib might have therapeutic effects by blocking Itk [102].

Inhibition of Itk by ibrutinib may constitute a novel non-steroidal treatment for allergy and other T-cell mediated hypersensitivities [103-106]. Itk is necessary for FcεRI aggregation and signaling in basophils. Stimulation of basophils with IgE, followed by treatment with ibrutinib reduced the expression of CD63, histamine, leukotriene C4 (LTC_4) and IL-4 secretion at an IC_{50} of 3-6 nM. Expression of CD203c, CD11b and the cytosolic calcium response was inhibited at an IC_{50} of 30-40 nM. The results might indicate that Btk is involved in IgE-mediated activation of human basophils [107].

Ibrutinib may inhibit B-cell activation and proliferation and to inhibit activation through IgG and FcεRI but not TLR4 [108]. Several studies have shown a role for Btk in macrophage activation through TLR4 [109]. The ability to block TLR signaling may be of benefit in RA as TLR signaling might contribute to the development of RA mediated by endogenous TLR ligands [109-110].

Effects of ibrutinib on the crosstalk between the BCR and Ror1 receptor tyrosine kinase

Ror1 receptor tyrosine kinase is expressed in several solid tumors and hematologic malignancies and has important roles in tumor cells survival, migration and metastasis [111-114]. Cross-communication between BCR and Ror1 signaling pathway has been recently described [115]. It has been shown that BCR and Ror1 modulate each other in a counterbalancing manner that is

important for tumor cell survival in acute lymphocytic leukemia (ALL) [115]. Downregulation of BCR by dasatinib and inactivation of Akt, as well as inhibition of Igα and Igβ, induced upregulation of Ror1 in ALL cells. Downregulation of both Ror1 and BCR induced dephosphorylation of Akt, inhibited cell growth and increased tumor cell killing. However, downregulation of either Ror1 or BCR alone did not induce the same effects, suggesting complimentary effects. The data also indicated an intracellular link (PI3K/Akt) between the pre-BCR and Ror1 signaling pathways, in which Btk was of importance [115].

Based on the current findings, an interesting model has been suggested. According to this model, during the development of B cells in bone marrow, B cells at the pre-BII stage upregulate Ror1 and the pre-BCR complex. Pre-BCR activates the Akt signaling pathway to induce B-cell proliferation and inhibit differentiation. To maintain B-cell development, the complex of pre-BCR is internalized and surrogate light chain is replaced by κ or λ chains. Surface expression of the mature BCR complex promotes B-cell differentiation. Signaling by the mature BCR activates the BLNK/PLCγ2 and this complex inhibits Akt activation and promotes cell maturation. At this stage, loss of active Akt is deleterious for cells, but transient expression of Ror1 provides an alternative mechanism for cell survival through activation of the Mek/Erk signaling pathway. Ror1 expression drives partial reactivation of Akt and provides additional signals for cell survival. In t(1;19) pre-B ALL cells, the E2A-PBX1 fusion protein contributed to B-cell arrest at the pre-BII stage and both the pre-BCR complex and Ror1 expression provided sustained survival signals for the malignant B-cell progenitors [115-116]. These data might suggest that the common pathways, which are used by BCR and Ror1 are downstream of Btk and might be affected by ibrutinib.

Part of the effects of ibrutinib on Btk and inactivation of BCR signaling might indirectly be due to downregulation of Ror1. However, downregulation of BCR may induce upregulation of Ror1 expression and activation in ibrutinib treated cells. This phenomenon may compromise the effects of ibrutinib and induce ibrutinib resistance. Combination of Ror1 inhibitors [3, 114] and ibrutinib might have a synergic anti-tumor effects. Further investigations are warranted to evaluate these effects.

Ibrutinib side-effects and tumor cells resistance

Ibrutinib is well tolerated. Side effects are mainly grade 1-2, including dyspnea, rash, nausea, diarrhea, fatigue, upper respiratory tract infections, and edema. These side effects seldom require therapeutic intervention [72, 117]. Treatment delays or discontinuation due to side effects of ibrutinib are rare. Major toxicities, including grade 3 and 4 have been observed in CLL and MCL and were mostly infections, as pneumonias, but the relation is uncertain [72, 117]. Ibrutinib does not seem to induce myelosuppression [93, 118]. Ibrutinib has been shown to affect platelet functions and based on preclinical observations it is suggested that Btk might play a role in platelet aggregation [119] by transferring signals from the platelet glycoprotein Ib (GPIb). Btk is also important for signaling via the collagen receptor GPVI in platelets [120]. An increased risk of bleeding has been observed in some patients [121]. Notably, patients with XLA, who have defective Btk, do not seem to have an increased risk for bleeding events [122].

Resistance to SMIs through mutations (off-target) during treatment may occur. Mutations that change drug binding to the target have been observed in several cancers, including EGFR [123-124] and anaplastic lymphoma kinase (Alk) [125] in lung cancer, fms-like tyrosine kinase 3 (FLT3) in acute myeloid leukemia (AML) [126] and Kit in gastrointestinal stromal tumors [127]. Approximately 30% of CLL and MCL patients exhibit resistance against ibrutinib [29, 128]. Recurrent mutations of Btk and its substrate PLCγ2 will induce resistance in a subset of CLL and MCL patients who have been well responded to the treatment or get prolonged treatment with ibrutinib relapse. A cysteine-to-serine (Btk^{C481S}) mutation in Btk at the binding site of ibrutinib has been reported [129-130]. The mutation changed the covalent binding between the ibrutinib and Btk (decreased binding affinity) creating a protein that is reversibly inhibited by ibrutinib. Moreover, three mutations in PLCγ2 (S707Y, R665W, and L845F) (downstream of Btk) induced tumor cell resistance to ibrutinib. These mutations are gain-of-function mutations leading to constant activation of BCR that is independent of Btk [130].

Concluding remarks

Immunomodulatory drugs are emerging as new and valuable generation of SMIs. These molecules target special kinases and have multiple therapeutic effects in various disorders. This

generation of first-in-class drugs may have several benefits for the targeted therapy of cancer, autoimmune and infectious diseases. Ibrutinib is an example of this new generation. The drug targets B cells by binding to Btk, which is upregulated in most B-cell malignancies. However, due to the multi-targeted characteristics, it also binds to Itk in Th cells and inhibits proliferation and differentiation of Th2 cells. Th1 cells are rescued from the inhibitory effects of ibrutinib through the activation of Rlk compensatory signaling, which is not expresses in Th2 cells. Ibrutinib may also emerge as an immune modulating agent. There is to our knowledge not yet any clinical trial testing the effects of ibrutinib in autoimmune and allergic disorders or infectious diseases. Ibrutinib might, however, be an effective inhibitor of autoreactive B cells and inhibits Th2 cells to prevent progression of B-cell related disorders. Further investigations are warranted to study the effects on the immune system and reveal other unknown therapeutic effects of ibrutinib.

Competing interest

Anders Österborg has received grants for clinical trials on ibrutinib and honoraria for scientific lectures from Janssen Pharmaceuticals. The other authors have no relevant affiliation or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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Figures legend:

Figure 1. Tec family structure. Members of Tec family kinases are schematically depicted showing the structural domains.

Figure 2. BTK structure and associated intracellular proteins. BTK is the central key tyrosine kinase in BCR signaling pathway. Several intracellular molecules bind to different domains of BTK and transduce signals.

Figure 3. Schematic presentation of the BCR signaling pathway and the targets for different small molecule inhibitors (SMI). The inhibitors prevent phosphorylation and activation of several enzymes involved in survival, proliferation and differentiation of B cells.